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A Novel α-Glucosidase of the Glycoside Hydrolase Family 31
from *Aspergillus sojae*

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Abstract: We characterized an α-glucosidase belonging to the glycoside hydrolase family 31 from *Aspergillus sojae*. The α-glucosidase gene was cloned using the whole genome sequence of *A. sojae*, and the recombinant enzyme was expressed in *Aspergillus nidulans*. The enzyme was purified using affinity chromatography. The enzyme showed an optimum pH of 5.5 and was stable between pH 6.0 and 10.0. The optimum temperature was approximately 55 °C. The enzyme was stable up to 50 °C, but lost its activity at 70 °C. The enzyme acted on a broad range of maltooligosaccharides and isomaltooligosaccharides, soluble starch, and dextran, and released glucose from these substrates. When maltose was used as substrate, the enzyme catalyzed transglucosylation to produce oligosaccharides consisting of α-1,6-glucosidic linkages as the major products. The transglucosylation pattern with maltopentaose was also analyzed, indicating that the enzyme mainly produced oligosaccharides with molecular weights higher than that of maltopentaose and containing continuous α-1,6-glucosidic linkages. These results demonstrate that the enzyme is a novel α-glucosidase that acts on both maltooligosaccharides and isomaltooligosaccharides, and efficiently produces oligosaccharides containing continuous α-1,6-glucosidic linkages.

Key words: α-glucosidase, *Aspergillus*, glycoside hydrolase family 31, transglucosylation

INTRODUCTION

α-Glucosidase (EC 3.2.1.20, α-D-glucoside glucohydrolase) is a typical exo-type glycosidase that hydrolyzes α-1,4-glucosidic linkages to release α-glucose from non-reducing ends of substrates. α-Glucosidase plays a key role in the metabolism of starch and glycogen. In the CAZy classification, enzymes numbered EC 3.2.1.20 are found in the glycoside hydrolase (GH) families 4, 13, 31, 63, 97, and 122, and the majority of α-glucosidases are classified into GH13 and GH31.

The GH31 family includes a wide variety of enzymes, such as α-xylosidases (EC 3.2.1.177), isomaltosyltransferases (EC 2.4.1.-), maltase/glucoamylases (EC 3.2.1.20 and 3.2.1.3), and sulfoquinovosidases (EC 3.2.1.-), in addition to α-glucosidases.[1] α-Glucosidases belonging to family GH31 are also diverse in enzymatic properties. Many GH31 α-glucosidases catalyze transglucosylation as well as hydrolysis, and some fungal GH31 α-glucosidases produce saccharides containing α-1,2-, α-1,3-, and α-1,6-glucosidic linkages. For instance, α-glucosidases from some *Acremonium* species produce oligosaccharides containing α-1,3-glucosidic linkages,[2)3)] and *Paecilomyces lilacinus* α-glucosidase produces oligosaccharides containing both α-1,2- and α-1,3-glucosidic linkages.[4]

The whole genome of *Aspergillus* species has recently been sequenced, showing that multiple genes encoding GH31 enzymes are present. *Aspergillus nidulans, Aspergillus niger,* and *Aspergillus oryzae* possess 10, 7, and 10 genes for GH31 enzymes, respectively, according to the CAZy database. We expect that some of these genes encode α-glucosidases with strong transglucosylation activity, and, in fact, a GH31 α-glucosidase from *A. niger*, AnigAgdA, has been reported to catalyze transglucosylation.[5)6)]

In this study, we focused on a GH31 α-glucosidase from *Aspergillus sojae*, AsojAgdL. *A. sojae* is a fungus traditionally used in Japan to produce fermented food, and a draft genome sequence of *A. sojae* is available.[7)] AsojAgdL is comprised of 951 amino acid residues and shows 31 %

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identity to AnigAgdA. Here we overexpressed AsojAgdL and AnigAgdA in *A. nidulans*, and their kinetic parameters were determined and compared. The transglucosylation products of AsojAgdL were also analyzed. The results show that AsojAgdL is a novel α-glucosidase that can produce transglucosylation products with a high ratio of α-1,6-glucosidic linkages. Using this enzyme, an industry-scale production of new kinds of isomaltooligosaccharides is possible.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from WAKO Pure Chemical (Japan), unless otherwise stated. Isomaltotetraose, isomaltopentaose, and isomaltohexaose were purchased form Seikagaku Corporation (Japan). Nigerose was purchased from Cosmobio (Japan) and isomaltotriose from Sigma-Aldrich (USA). 3^2-*O*-α-D-Glucosyl-maltose and isomaltotriosyl-glucose were prepared as previously described.[3][8]

Microorganisms. *A. sojae* NBRC4239 and *A. niger* NBRC4066 were obtained from the NITE Biological Resource Center (Japan). *A. oryzae* RIB40 was obtained from the National Research Institute of Brewing (Japan). *A. nidulans* ATCC38163 was obtained from the American Type Culture Collection (USA).

α-Glucosidase gene cloning and expression vector construction. The sequence of the *AsojAgdL* gene was identified in the whole genome sequence of *A. sojae* NBRC4239 (accession no. BACA00000000) using the homolog gene sequence of *A. oryzae* (accession no. XP_001825390). Phusion Hot Start II DNA polymerase (Thermo Fisher Scientific, USA) was used in PCRs, and the oligonucleotide primers used are shown in Table S1 (see J. Appl. Glycosci. Web site). To express the *AsojAgdL* gene under the control of the *A. oryzae TEF1* gene promoter, an expression plasmid was constructed as follows. An 0.8 kb fragment containing the *TEF1* gene promoter was amplified from genomic DNA of *A. oryzae* RIB40 using the primer pair promoter1/promoter2. The *AsojAgdL* gene without the stop codon was amplified from genomic DNA of *A. sojae* NBRC4239 using the primer pair AgdL1/AgdL2. An 0.3 kb fragment of the α-glucosidase terminator containing 10 × His-tag and a stop codon was amplified from genomic DNA of *A. sojae* NBRC 4239 using the primer pair terminator 1/terminator 2. The three PCR products and the HindIII/KpnI-digested pPTRII (Takara Bio) were joined in a four-piece In-Fusion reaction using the In-Fusion HD Cloning Kit (Takara Bio). Cloning of α-glucosidase from *A. niger* (AnigAgdA) was similarly carried out, except that primers AgdA1, AgdA2, terminator3, and teminator4 and genomic DNA from *A. niger* NBRC4066 were used instead.

Transformation. *A. nidulans* ATCC38163 was transformed with expression vectors containing *AsojAgdL* and *AnigAgdA* according to the method described by Gomi *et al.*[9]

Sequence analysis. The cDNA of *AsojAgdL* was obtained using reverse transcriptase-PCR. Total RNA of recombinant DNA-containing cells was extracted and purified using RNeazy Plant Mini Kit (Qiagen, Netherland). First-strand cDNA was synthesized using oligo (dT) primer and subjected to PCR using primer pair AgdL3/AgdL2. The PCR product was cloned into PCR2.1 TOPO (Invitrogen, USA), sequenced, and the amino acid sequence of AsojAgdL confirmed. Confirmation of the DNA sequence of *AnigAgdA* was carried out by sequence analysis of the expression vector prepared as described above.

Enzymatic assays. α-Glucosidase activity was determined by incubating the enzyme with 20 mM maltose in 20 mM MES buffer (pH 6.0) for 30 min at 40 °C. The reaction was terminated by adding 10 % oxalic acid and boiling for 10 min. Released glucose was measured using Glucose CII Test Wako (Wako Pure Chemical). One unit of α-glucosidase activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of maltose per min. Hydrolysis of various substrates by AsojAgdL or AnigAgdA in various concentrations were examined. AsojAgdL was incubated with substrates in 20 mM MES buffer (pH 6.0) for 60 min at 40 °C, and AnigAgdA was incubated with substrates in 20 mM acetate buffer (pH 4.0) for 60 min at 40 °C. The reaction was terminated by adding 10 % oxalic acid and boiling for 10 min. Released glucose was measured using Glucose CII Test Wako. Kinetic parameters were calculated using nonlinear regression analysis of KaleidaGraph (Synergy Software, USA). To estimate substrate inhibition, the K_i value was calculated using the following formula: $v = V_{max}[S]/(K_m+[S]+[S]^2/K_i)$.[10] Thermal and pH stabilities of AsojAgdL were determined by residual activity after incubation of enzyme solutions at various temperatures (from 40 °C to 70 °C) for 1 h and at various pH values (from 2.0 to 12.0) for 24 h at 4 °C, respectively. Phthalic acid-HCl buffer at pH 2.0 to 3.5, phthalic acid -NaOH buffer at pH 4.0 to 5.5, MES buffer at pH 5.5 to 7.0, MOPS buffer at pH 7.0 to 8.5, and CAPS buffer at pH 9.0 to 12.0 were used for pH adjustment.

Purification of recombinant α-glucosidase. For purification of recombinant AsojAgdL, transformed *A. nidulans* was cultivated aerobically at 37 °C for 4 days with rotary shaking at 180 rpm in 2-liter Erlenmeyer flasks, each containing 1 L of czapek-dox medium with 0.1 μg/mL pyrithiamine. Mycelia were separated by filtration with miracloth (Merk, Germany). A total of 100 g of mycelia was suspended in Tris-buffer (pH 7.4) with 20 mM imidazole and homogenized with Hiscotron (Nichion, Japan). Ground mycelia were centrifuged at 15,700 × G for 20 min. The resulting supernatant was used as the cell extract. The cell extract was applied to a His-Trap HP column (GE Healthcare, USA) and then eluted in 20 mM Tris buffer (pH 7.4) containing 0.5 M NaCl and 500 mM imidazole. The eluate was applied to a Hiload 16/60 Superdex 200 Prepgrade column (GE Healthcare) and then eluted in 20 mM MES buffer (pH 6.0). The active fractions were collected and used as purified enzyme. Purification of AnigAgdA was carried out using the same procedure up to the elution from His-Trap HP column, after which the process was considered complete.

Physical measurements. Protein concentration was determined using Quick Start Bradford Protein Assay (Bio-Rad, USA). The apparent molecular mass of the enzymes was determined using SDS-PAGE in 10–15 % gradient polya-

crylamide gels as described by Laemmli.[11] Benchmark Ladder (Invitrogen) was used as standard protein markers. The molecular mass of the native enzymes was determined using native-PAGE in 8–25 % gradient polyacrylamide gels as described by Davis.[12] HMW Marker Kit (GE Healthcare) was used as standard protein markers. The p*I* values of thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and albumin (66 kDa) present in the protein marker are 4.5, 4.5, 5.4, 5.0, and 4.9, respectively. The apparent p*I* of the enzymes was determined using isoelectric focusing on a Phastgel IEF 3–9 and the Phastsystem (GE Healthcare). Proteins were stained using Rapid CBB KANTO (Kanto Chemical, Japan).

Preparation of transglucosylation products. Transglucosylation products of AsojAgdL were synthesized using the purified enzyme. Using maltose as substrate, the enzyme was incubated with 30 % (wt/wt) maltose at 2 U/g DS (dissolved substrate) at pH 6.0, 40 °C, for 1, 2, 6, 12, 24, 48, and 72 h. Using maltopentaose as substrate, the enzyme was incubated with 30 % (wt/wt) maltopentaose at 8 U/g DS at pH 6.0, 40 °C, for 72 h. The reaction was terminated by boiling for 10 min. Transglucosylation products of AnigAgdA were synthesized using a commercially available enzyme, Transglucosidase L (Amano Enzyme, Japan). The enzyme was incubated with 30 % (wt/wt) maltopentaose at 8 U/g DS at pH 6.0, 40 °C, for 72 h. The reaction was terminated by boiling for 10 min.

HPLC analysis and purification of transglucosylation products. Transglucosylation products were analyzed using HPLC under the following conditions. For the degree of polymerization (DP) analysis, the HPLC system was used with an MCI GEL CK04S column (Mitsubishi Chemical, Japan) at 65 °C and a refractive index detector. To determine the DP of each peak, analysis was carried out in the same manner as in the DP analysis except that ELSD and LC MS were connected in parallel as detectors. The transglucosylation products were eluted using distilled water at a flow rate of 0.35 mL/min. For the structural isomer analysis, the HPLC system was used with a Unison UK-Amino (Imtact, Japan) column at 50 °C and a NQAD detector. The transglucosylation products were separated with a linear gradient under the following conditions; initial ratio of acetonitrile/water at 88 % and a flow rate of 0.4 mL/min, then decreased to 81 % for 30 min, then held at 81 % for 35 min, then decreased to 60 %, then held at 60 % for 10 min, and then increased back to 88 %. The fractionation of transglucosylation products were carried out using HPLC under the following conditions. The HPLC system was used with a Bio-Gel P2 (Bio-Rad) column at 60 °C and a refractive index detector. The transglucosylation products were eluted using distilled water at a flow rate of 9 mL/min.

Spectrometry. NMR spectra were obtained using a UNITY INOVA 500 (Varian, USA) with ^{1}H-NMR at 500 MHz. Chemical shifts are given in ppm using water as the standard.

MALDI-TOF MS analysis. MALDI-TOF mass spectra were obtained on a Autoflex Speed IC TOF/TOF (Bruker BioSpin, USA) using 2,5-dihydroxybenzoic acid as a ma-

trix. An aliquot of 1 μL of sample was mixed with 1 μL of 2,5-dihydroxybenzoic acid in TA30 (30:70 [vol/vol] acetonitrile:TFA 0.1 % in water) and dried at room temperature. The molar masses were analyzed in the positive reflectron mode.

Dextranase hydrolysis. Evaluation of transglucosylation products using dextranase hydrolysis was carried out with the following method. Transglucosylation products were hydrolyzed with 1 % (wt/wt) of 1000-fold diluted Dextranase L (Amano Enzyme) at 5 % (wt/wt) substrate concentration at pH 6.0, 50 °C, for 16 h, and the reaction was stopped with boiling. Analysis of the reactants was carried out in the same manner as the above-described method of DP analysis of transglucosylation products using HPLC.

Methylation analysis. The linkages of transglucosylation products were determined using methylation analysis as described by Cicanu and Kerek.[13] Gas chromatographic analysis was performed using GC 7890A (Agilent Technologies, USA) and a FID detector under the following conditions. Column: HP-5MS fused silica capillary (30 m × 0.25 mm ID; Agilent Technologies); column temperature: 100 °C for 1 min, then 280 °C for 30 min, and left to stand for 1 min; injection temperature: 280 °C; and eluent: helium.

RESULTS AND DISCUSSION

### Purification and biochemical characterization of AsojAgdL.

The recombinant AsojAgdL was purified using His-Trap affinity chromatography and gel filtration (Table 1). The purified AsojAgdL corresponded to two polypeptide chains with apparent molecular masses of 65 kDa and 76 kDa, respectively, on SDS-PAGE (Fig. 1A). Isoelectric focusing results indicate that, although the gel signal was smeared, the p*I* of AsojAgdL was approximately 5.2 (Fig. 1C), which is close to those of the molecular mass standards for native PAGE. The calculated p*I* from the amino acid sequence using ExPASy's ProtParam tool (https://web.expasy.org/protparam/) is 4.8, which is not significantly different from the results of isoelectric focusing. Native PAGE analysis of AsojAgdL showed a clear single polypeptide chain detected at 110 kDa (Fig. 1B), suggesting that it is a heterodimeric protein and that the single polypeptide chain was successfully cleaved during the heterologous expression in *A. nidulans*. As previously reported, *A. nidulans* α-glucosidase is also found as a heterodimer.[14] The amino acid sequence of AnigAgdA, predicted by the DNA sequence from *A. niger* ATCC4066 in the expression vector, was identical to its previously reported sequence.[15][16] The recombinant AnigAgdA was purified using His-Trap affinity chromatography. The purified enzyme corresponded to two polypeptide chains on SDS-PAGE (Fig. 2A). AnigAgdA is known to be a heterodimer, but is not separable using SDS-disc gel electrophoresis.[15] The reason is not clear, as the two components of the enzyme were separated using SDS-PAGE in this study. In isoelectric focusing, although the gel signal was smeared, two polypeptide chains were detected between p*I* standards of 5.20 and 6.55 (Fig. 2C),

Table 1. Purification of recombinant AsojAgdL from transformed *Aspergillus nidulans*.

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Cell extract	124.1	15.6	0.13	100
His-Trap HP	13.3	12.6	0.95	81
Hiload 16/60 Superdex 200 Prepgrade	0.4	1.9	5.38	12
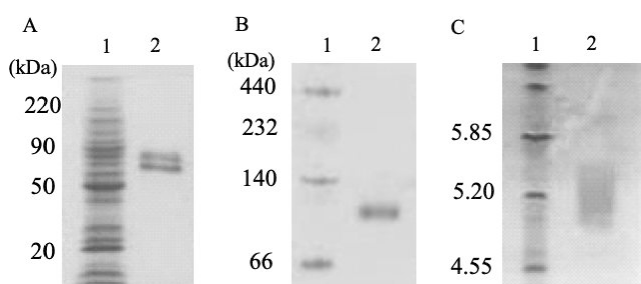
Fig. 1. Molecular mass and p*I* of purified AsojAgdL.
(A) SDS-PAGE of Benchmark Ladder (lane 1) and purified AsojAgdL (lane 2). (B) Native-PAGE of HMW marker kit (lane 1) and purified AsojAgdL (lane 2). (C) Isoelectric focusing using the Broad p*I* kit (lane 1) and purified AsojAgdL (lane 2).
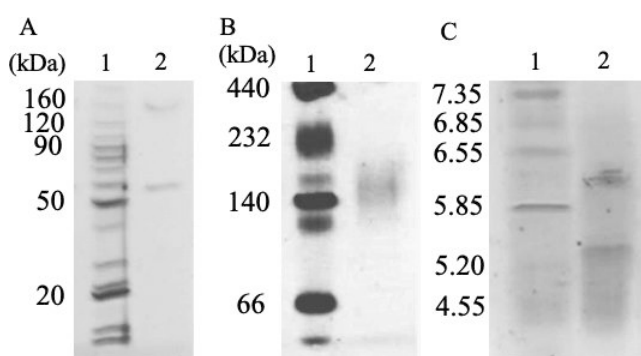
Fig. 2. Molecular mass and p*I* of purified AnigAgdA.
(A) SDS-PAGE of Benchmark Ladder (lane 1) and purified AnigAgdA (lane 2). (B) Native-PAGE of HMW marker kit (lane 1) and purified AnigAgdA (lane 2). (C) Isoelectric focusing using the Broad p*I* kit (lane 1) and purified AnigAgdA (lane 2).

which are close to those of molecular mass standards for native PAGE. The calculated p*I* from the amino acid sequence using ExPASy's ProtParam tool is 5.1, which is not significantly different from the results of isoelectric focusing. Native PAGE analysis of AnigAgdA showed a single polypeptide chain detected at 140 kDa (Fig. 2B).

The effects of pH and temperature on the activity and stability of the enzymes were examined. AsojAgdL showed an optimum pH of 5.5, and less than 10 % of activity was lost between pH 6.0 and 10.0. The optimum temperature was approximately 55 °C. Less than 10 % of activity was lost up to 50 °C, whereas 95 % of activity was lost at 70 °C.

Kinetic analysis of AsojAgdL.

To elucidate the enzymatic properties of AsojAgdL, ki-

netic parameters of the enzyme for various saccharides were determined, and the values were compared to those of AnigAgdA. The activities of both AsojAgdL and AnigAgdA were measured using the amount of glucose released from the substrates. No activity for trehalose was detected with either enzyme. The activities for maltooligosaccharides, isomaltooligosaccharides, kojibiose, nigerose, soluble starch, and dextran are shown in Table 2.

When maltooligosaccharides were tested as substrates, the enzymatic activities of both AsojAgdL and AnigAgdA were inhibited by high substrate concentrations (approximately 2 mM or higher). At lower substrate concentrations, the enzymatic reaction fitted well to the Michaelis-Menten kinetic model (Fig. 3A), whereas the entire curve of substrate concentration versus initial velocity obeyed the substrate inhibition kinetics (Fig. 3B). Therefore, k_0 and K_m values were determined using only low substrate concentration ranges, and K_i values were calculated using the entire curves of the plots (Table 3). In contrast, no substrate inhibition was observed for isomaltooligosaccharides, kojibiose, or nigerose in the reactions with both AsojAgdL and AnigAgdA (Fig. 3C).

Both enzymes exhibited a broad substrate specificity for oligosaccharides, but the substrate preferences were different. The k_0/K_m values of AsojAgdL for the substrates listed in Table 2 were similar; the lowest value was 1.4 mM s^{-1} (for kojibiose) and the highest value was 17.7 mM s^{-1} (for maltohexaose). Although AsojAgdL preferred maltooligosaccharides and the k_0/K_m values for maltotriose to maltohexaose were 11.0–17.7 mM s^{-1}, the enzyme also acted on isomaltooligosaccharides; the k_0/K_m values for isomaltose to isomaltohexaose were approximately 3 mM s^{-1}. In contrast, AnigAgdA was highly specific for maltooligosaccharides, and the k_0/K_m values for maltotriose to maltohexaose were approximately 30 mM s^{-1}. The enzyme, however, showed low activity for isomaltooligosaccharides, especially low activity was observed for isomaltopentaose and isomaltohexaose compared to maltose. Using maltooligosaccharide, substrate inhibition was observed for both AsojAgdL and AnigAgdA, but AsojAgdL was significantly lower in K_i value than AnigAgdA except for maltose. Both AsojAgdL and AnigAgdA acted on soluble starch. In contrast, AsojAgdL hydrolyzed dextran but AnigAgdA did not.

AsojAgdL showed 82 % amino acid sequence identity to *A. nidulans* AgdB. For the latter enzyme, it was previously reported that the most favored substrate was maltotriose, and the k_0/K_m values for maltotetraose and maltopentaose decreased in this order. In addition, soluble starch was the least-favored substrate.[14] For AsojAgdL, on the other hand, longer maltooligosaccharides were favored, and it also acted on soluble starch. These results indicate that AsojAgdL has different substrate specificities than *A. nidulans* AgdB.

Transglucosylation by AsojAgdL with maltose as substrate.

The transglucosylation products of AsojAgdL when maltose was used as substrate were analyzed using HPLC (Fig. 4), and the time course of isomaltooligosaccharide composition is shown in Fig. 5. A trisaccharide panose (Glc-

Table 2. Kinetic parameters of AsojAgdL and AnigAgdA for hydrolysis of various substrates.

Substrates	AsojAgdL			AnigAgdA		
	K_m (mM)	k_0 (s^{-1})	k_0/K_m (mM^{-1}s^{-1})	K_m (mM)	k_0 (s^{-1})	k_0/K_m (mM^{-1}s^{-1})
Trehalose	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]
Maltose	2.2±0.3	8.5±0.4	3.9	3.2±0.8	52.3±6.1	16.5
Kojibiose	2.1±0.3	2.9±0.1	1.4	5.2±0.1	16.8±0.1	3.2
Nigerose	2.5±0.4	5.1±0.2	2.1	13.0±0.3	34.0±0.4	2.6
Isomaltose	2.0±0.3	4.9±0.3	2.4	5.0±0.1	20.6±0.2	4.1
Maltotriose	2.0±0.0	22.1±0.3	11.0	2.0±0.4	62.8±5.8	31.7
Maltotetraose	1.6±0.1	22.1±1.3	14.1	1.2±0.2	47.4±3.3	39.4
Maltopentaose	0.9±0.1	15.3±1.5	17.0	1.1±0.1	40.4±1.3	36.3
Maltohexaose	0.9±0.1	15.9±1.1	17.7	1.1±0.1	43.1±2.0	38.7
Soluble starch[d]	0.4±0.1	9.3±1.1	24.4	3.0±0.5	184.3±16.5	62.0
Isomaltotriose	0.5±0.1	3.6±0.1	7.0	11.2±0.4	38.3±0.7	3.4
Isomaltotetraose	2.4±0.8	5.9±0.6	2.4	32.8±3.5	43.2±3.0	1.3
Isomaltopentaose	0.9±0.1	3.6±0.1	4.1	ND[b]	ND[b]	0.6[c]
Isomaltohexaose	1.1±0.2	3.5±0.1	3.1	ND[b]	ND[b]	0.5[c]
Dextran[d]	0.9±0.2	2.7±0.2	3.1	ND[b]	ND[b]	0.4[c]

K_m and k_0 values are shown with standard errors. [a]Not determined because of low activity. [b]Not determined because the enzyme was not saturated with the substrate [c]The k_0/K_m value was determined from initial velocities (v) at sufficiently low substrate concentration ($\{S\}$) based on the relationship $k_0/K_m = v/(\{S\}\{E\})$ [d]Weight average molecular weight of soluble starch and dextran were 8,778 and 11,096, respectively, as determined by gel permeation chromatography.
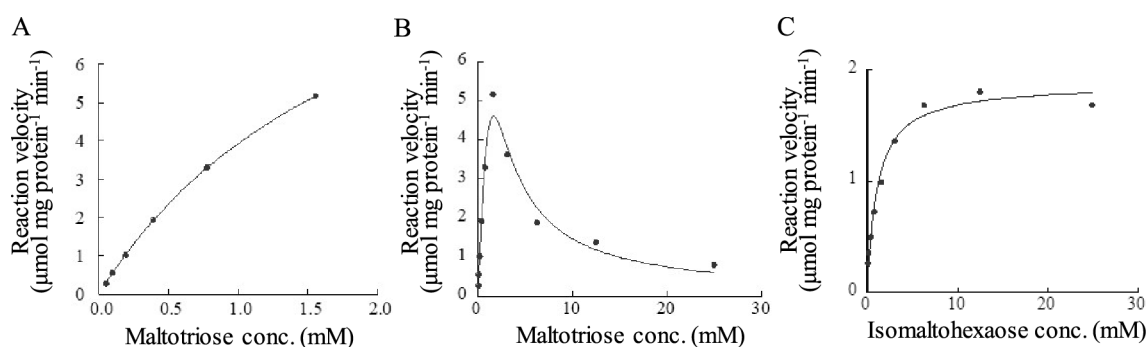
Fig. 3. Kinetic analysis of AsojAgdL.
Plots of reaction velocity versus substrate concentration for hydrolysis of (A) maltotriose at 0-1.6 mM, (B) maltotriose at 0–25 mM, and (C) isomaltohexaose at 0–25 mM are shown.

α-1,6-Glc-α-1,4-Glc) appeared first, and its levels peaked at 6 h. A tetrasaccharide isomaltotriosyl-glucose (Glc-α-1,6-Glc-α-1,6-Glc-α-1,4-Glc) appeared next and its levels peaked at 12 h. Both panose and isomaltotriosyl-glucose then decreased. Isomaltose, isomaltotriose, and isomaltotetraose, which contain no α-1,4-glucosidic linkages, appeared in this order and their levels equilibrated after 24 h. A possible mechanism to produce isomaltooligosaccharides is shown in Fig. 6. AsojAgdL acts on maltose catalyzing both transglucosylation and hydrolysis to produce panose and glucose. In the early stages of the reaction, sugar production mainly occurs via Route A, and panose and isomaltotriosyl-glucose accumulate. After maltose is completely consumed, panose and isomaltotriosyl-glucose are hydrolyzed, and Route B becomes the major sugar production pathway. The amount of glucose reached high levels, and the overall sugar composition, which contained mainly isomaltose, isomaltotriose, and isomaltotetraose, was almost in equilibrium. The fraction of pentasaccharides after 72 h of the reaction was collected and analyzed using ^{1}H-NMR,

Table 3. K_i values of AsojAgdL and AnigAgdA for maltoorigosaccharides.

Substrates	K_i (mM)	
	AsojAgdL	AnigAgdA
Maltose	–[a]	12.0±3.6
Maltotriose	0.0083±0.59	3.8±0.7
Maltotetraose	0.0029±0.34	7.2±5.0
Maltopentaose	0.29±0.37	19.6±2.2
Maltohexaose	0.14±0.29	30.8±7.0

Values are shown with standard errors. [a] Not determined because the activity was not inhibited.

and the ratio of α-1,6-glucosidic linkage was 81 %. Considering that the ratio of α-1,6-glucosidic linkages below pentasaccharides occupied a large part at 72 h of reaction as described above, the α-1,6-glucosidic linkage is dominant in the entire range of transglucosylation products from maltose.

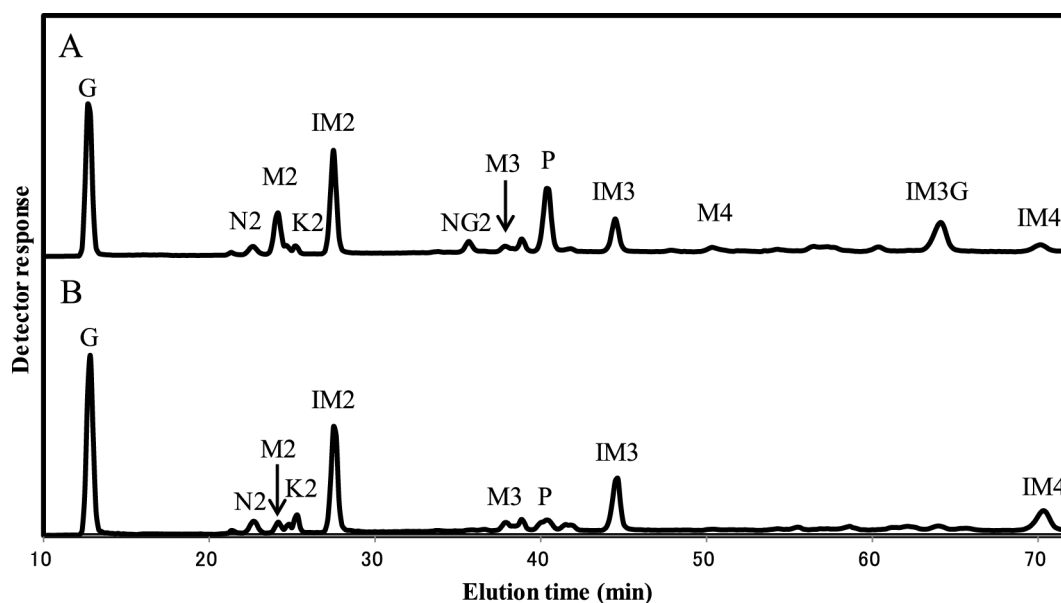The time course of transglucosylation products of Aso-

Fig. 4. HPLC patterns of transglucosylation products of AsojAgdL using maltose as substrate.
(A) 12 h of reaction; (B) 72 h of reaction. G, glucose; N2, nigerose; M2, maltose; K2, kojibiose; IM2, isomaltose; NG2, 3^2-*O*-α-D-glucosyl-maltose; M3, maltotriose; P, panose; IM3, isomaltotriose; M4, maltotetraose; IM3G, isomaltotriosyl-glucose; IM4, isomaltotetraose.

jAgdL are summarized in Table 4. Although isomaltooligo-saccharides were the main products, the enzyme also produced other saccharides. The trisaccharide 3^2-*O*-α-D-gluco-syl-maltose and a small amount of nigerose were produced in the early and later stages of the reaction, respectively. These results indicate that AsojAgdL can produce α-1,3-glucosidic linkages. A small amount of kojibiose was produced in the later stages of the reaction, showing that Aso-jAgdL also produced a small amount of α-1,2-glucosidic linkages. The levels of maltotriose increased once in the early stages of reaction but then decreased, and maltote-traose levels showed a modest increase throughout the reaction. The results suggest that AsojAgdL produces α-1,4-glucosidic linkages, but the product is used as a glucosyl donor.

Transglucosylation by AsojAgdL with maltopentaose as substrate. In the DP analysis of transglucosylation products produced by the reaction of AsojAgdL with maltopentaose, oligosaccharides with a higher molecular weight than that of the substrate were detected. The HPLC chart after 72 h of reaction is shown in Fig. 7A. To estimate the DP of each peak, MS analysis was performed. The peaks at the retention times 29, 24, 21, 19, 17, and 15 min were identified as DP1, DP2, DP3, DP4, DP5, and DP6, respectively. However, analysis of DP6 was difficult because peaks overlapped. The molecular weights of 50 % of the transglucosylation product were larger than those of DP 5. The maximum molecular weight of the transglucosylation product was detected to be DP12 in the MALDI-TOF MS analysis (Fig. S1; see J. Appl. Glycosci. Web site).

The transglucosylation product was hydrolyzed with Dextranase L, and the percentage of transglucosylation products lager than DP5 decreased from 50 to 37 % (Fig. 7B). Dextranase (EC 3.2.1.11) derived from a filamentous fungus, used in dextranase hydrolysis in this work, is known to hydrolyze dextran and isomaltooligosaccharides
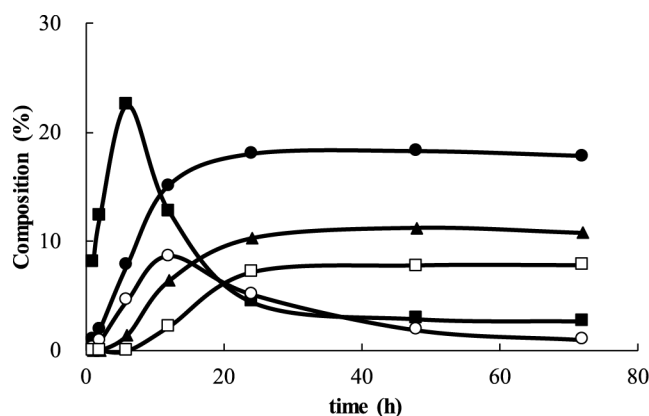

Fig. 5. Time course of transglucosylation product composition of AsojAgdL using maltose as substrate.
Closed circle, isomaltose; closed square, panose; closed triangle, isomaltotriose; open circle, isomaltotriosyl-glucose; open square, iso-maltotetraose.
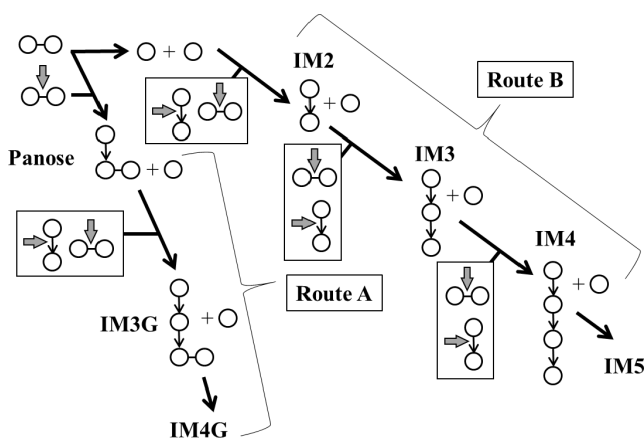

Fig. 6. Predicted routes of isomaltooligosaccharide production.
Circle, glucose; –, α-1,4-glucosidic linkage; ↓, α-1,6-glucosidic linkage; bold gray arrow, point of enzymatic attack; IM2, isomaltose; IM3, isomaltotriose; IM3G, isomaltotriosyl-glucose; IM4, isomaltote-traose; IM4G, isomaltotetraoosyl-glucose; IM5, isomaltopentaose.

Table 4. Time course of structural isomer composition of transglucosylation products of AsojAgdL using maltose as substrate.

		Time course (h)						
		1	2	6	12	24	48	72
		Composition (%)						
DP1	glucose	5.3	8.3	18.3	23.4	25.4	26.7	27.9
DP2	nigerose	0.0	0.0	0.6	1.5	2.1	2.2	2.3
	maltose	76.8	65.4	25.2	7.6	3.7	3.0	2.7
	kojibiose	0.0	0.0	0.0	1.1	1.9	2.2	2.4
	isomaltose	1.0	1.9	7.9	15.1	18.0	18.2	17.8
	unknown	1.1	0.9	0.7	0.3	0.0	0.0	0.4
DP3	3^2-*O*-α-D-glucosyl-maltose	2.1	3.3	5.4	1.8	0.3	0.0	0.0
	maltotriose	4.4	5.6	5.0	1.4	1.1	1.5	1.9
	panose	8.1	12.3	22.5	12.8	4.5	2.9	2.7
	isomaltotriose	0.0	0.0	1.4	6.4	10.3	11.3	10.8
	unknown	0.6	0.9	1.8	2.2	2.8	3.0	3.0
DP4	maltotetraose	0.2	0.4	0.6	0.4	0.4	0.4	0.0
	isomaltotriosyl-glucose	0.3	0.9	4.6	8.7	5.2	1.9	1.0
	isomaltotetraose	0.0	0.0	0.0	2.2	7.2	7.8	7.8
	unknown	0.0	0.0	4.2	5.4	2.2	3.2	3.7
DP5+		0.0	0.0	1.8	9.7	14.9	15.7	15.5
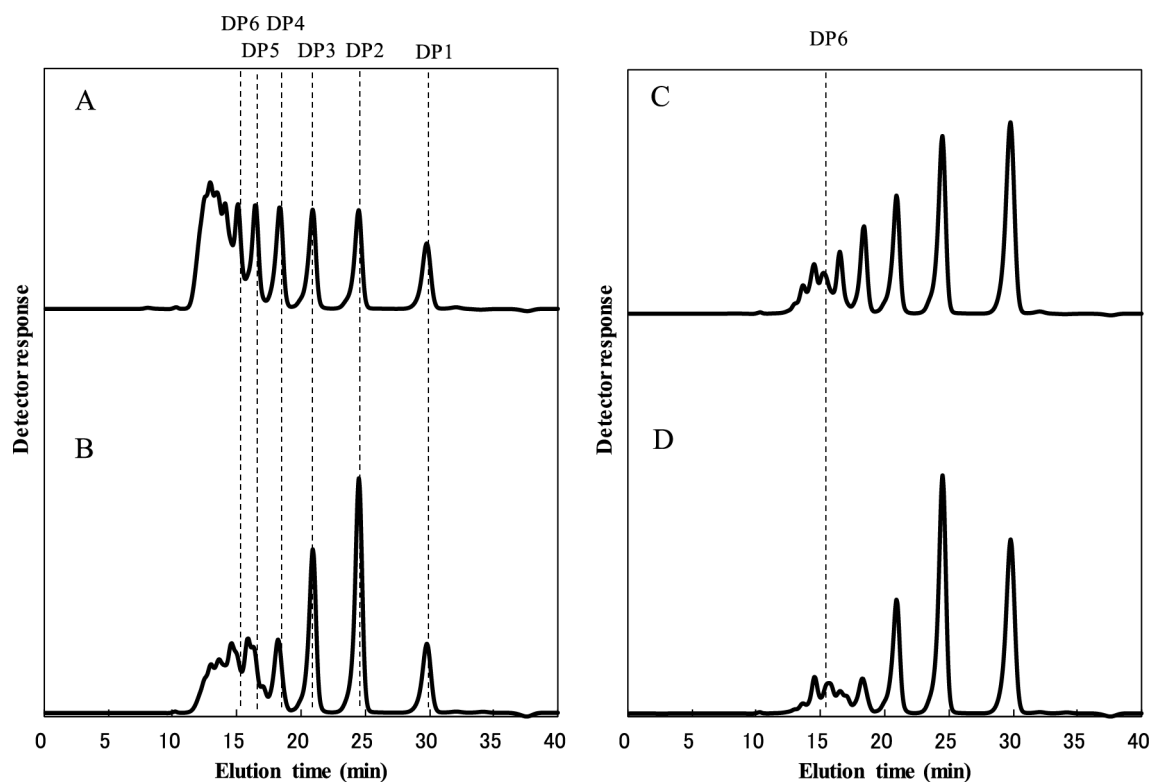
Fig. 7. HPLC chromatogram of transglucosylation products and its hydrolysate with dextranase.
(A) Transglucosylation product of AsojAgdL after 72 h of reaction using maltopentaose as substrate. (B) Hydrolysate of the transglucosylation product of (A) with dextranase. (C) Transglucosylation product of Transglucosidase L after 72 h of reaction using maltopentaose as substrate. (D) Hydrolysate of the former transglucosylation product of (C) with dextranase.

having longer α-1,6-glucosidic linkages than isomaltotriose and to mainly produce isomaltose, isomaltotriose, and a small amount of glucose.[17)18)19)] Therefore, AsojAgdL synthesized three or more continuous α-1,6-glucosidic linkages.

The fraction of oligosaccharides larger than DP5 in the transglucosylation product was collected and measured us-ing methylation analysis (Table 5). The dominant linkage was the α-1,6-glucosidic linkage, and most of the rest was the α-1,4-glucosidic linkage. Considering the transglucosylation path from maltose, the results suggest that this fraction consisted of α-1,4-glucosidic linkages at the reducing end and α-1,6-glucosidic linkages at non-reducing end. The ratio of α-1,6-glucosidic to α-1,4-glucosidic linkages was

Table 5. Methylation analysis of transglucosylation product of AsojAgdL larger than maltopentaose after 72 h of reaction.

Type of glucosidic linkage	Composition (%)
Terminal	16.1
1–3	4.8
1–4	25.0
1–6	44.5
1–2, 1–4	2.3
1–4, 1–6	2.9
1–3, 1–6	4.4
Others	-

approximately 2 to 1. Small amounts of α-1,2- and α-1,3-glucosidic linkages were also formed.

Similarly, Transglucosidase L, whose main activity is derived from AnigAgdA,[15)16)] reacted with maltopentaose, and the transglucosylation products were analyzed. The proportion of transglucosylation product larger than DP5 was 16 % after 72 h of reaction and decreased to 12 % after dextranase hydrolysis (Fig. 7C and 7D). The reduction was 4 %, which was one-third of that of AsojAgdL. Therefore, AsojAgdL is a completely different enzyme from AnigAgdA in its ability to generate oligosaccharides that have continuous α-1,6-glucosidic linkages. α-Glucosidases from *A. niger*, *A. nidulans*, *A. oryzae* are known to produce isomaltoorigosaccharides with short chains of α-1,6-glucosidic linkages such as isomaltose, isomaltotriose, and panose,[6)14)20)] but α-glucosidases that produce continuous α-1,6-glucosidic linkages have not been reported. It has been reported that isomaltooligosaccharides has the function of increasing the number of human intestinal bifidobacteria, and that trisaccharides of isomaltooligosaccharides have a stronger effect than disaccharides on the growth of bifidobacteria in human intestine.[21)] It is presumed from the present report that saccharides produced by AsojAgdL are also expected to have high prebiotic effects.

In conclusion, we characterized a novel α-glucosidase, AsojAgdL, that hydrolyzes both maltooligosaccharides and isomaltooligosaccharides. AsojAgdL also has a strong transglucosylation ability to produce oligosaccharides that contain a large number of continuous α-1,6-glucosidic linkages. It is expected that new prebiotic materials can be produced in an industrial scale using AsojAgdL.

CONFLICTS OF INTEREST

The authors declare no conflict of interests.

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